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Intranasal Immunization with Mumps Virus DNA Vaccine Delivered by Influenza Virosomes Elicits Mucosal and Systemic Immunity

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To improve the efficiency of liposome-mediated DNA transfer as a tool for gene therapy or vaccinology, we have further developed a new delivery system based on the modified immunopotentiating reconstituted influenza virus (IRIV). In this study, we engineered a plasmid DNA vector expressing the mumps virus hemagglutinin or the fusion protein. The administration of this DNA vaccine delivered by influenza virosomes, in combination with the mucosal adjuvant Escheriagen via the intranasal route, was efficient for inducing an immune response, both mucosally and systemically, in mice. The production of IgG2a mumps virus-specific antibodies and the secretion of interleukin 10 (IL-10) by antigen-specific T cells indicated that not only Th1 but also Th2 responses were induced by this DNA vaccine formulation. These results suggest that cationic virosomes in combination with Escheriagen may have great potential as an efficient delivery system for intranasal DNA immunization and provide an immune barrier at the mucosal sites.

Key Words: influenza virosome; mumps virus; DNA vaccine.

INTRODUCTION

Mumps virus is a member of the Paramyxoviridae family of the paramyxovirus genus. Parotitis is the most common symptom of the disease, but the incidence of meningitis following natural mumps infection has been estimated to occur in about 10% of all cases (Furesz and Contreras, 1990). Beginning in 1968, the widespread use of the live attenuated mumps virus vaccine was followed by a decrease in the incidence of mumps; however, cases of infection after vaccination have still been occurring (Boulianne *et al.,* 1995; Brown *et al.,* 1991; Forsey *et al.,* 1992; German *et al.,* 1996; Jonville-Bera *et al.,* 1996; Nalin, 1992; Sugiura and Jamada, 1991). Lack of clinical protection has been attributed to primary vaccine failure, which occurs in persons who do not seroconvert after vaccination or to a waning vaccine-induced immunity (Briss *et al.,* 1994).

In the present study we investigated the efficacy of a mumps DNA vaccine intranasally delivered by virosomes and administered to mice. Virosomes comprising cationic lipids and fusion active influenza hemagglutinin protein appear to be a very efficient delivery system for nucleic acid vaccines (Cryz and Glück, 1998; Waelti and Glück, 1998). The use of DNA vaccines associated with virosomes avoids the complex physicochemical problems associated with the use of adjuvants and may also result in *in vivo* antigen presentation of the encoded epitopes in a manner similar to the presentation of the epitope that would occur following natural infection. In this investigation we combined the virosomal carrier system with the mucosal adjuvant Escheriagen (*E. coli* heat-labile toxin; Swiss Serum and Vaccine Institute, Berne). Here we show that the priming of mice with influenza virosomes and Escheriagen before vaccination enhanced the humoral response. The results obtained show that specific s-IgA antibodies are produced in the mucosal layer of the respiratory tract and, in addition, circulating IgG antibodies are also produced, indicating that both systemic and distal mucosal responses are induced in intranasally immunized mice.

RESULTS AND DISCUSSION

Plasmid immunogens and antigen expression

We engineered two plasmids, GC9 (MuV HN) and GC23 (MuV F), to determine whether they could elicit protective immunity against MuV. They were designed to express the full-length, membrane-anchored MuV hemagglutinin and the fusion protein. Before testing them in mice, they were tested in transfection assays *in vitro.* Naked DNA, or DNA entrapped in influenza virosomes, was able to express the antigens in Vero cells, as shown by immunofluorescence tests (Fig. 1). The increased expression of MuV proteins *in vitro* by transfection of mixtures of DNA with virosomes as compared with transfection of DNA alone was then confirmed by immunoprecipitation assay, as shown in Fig. 2.

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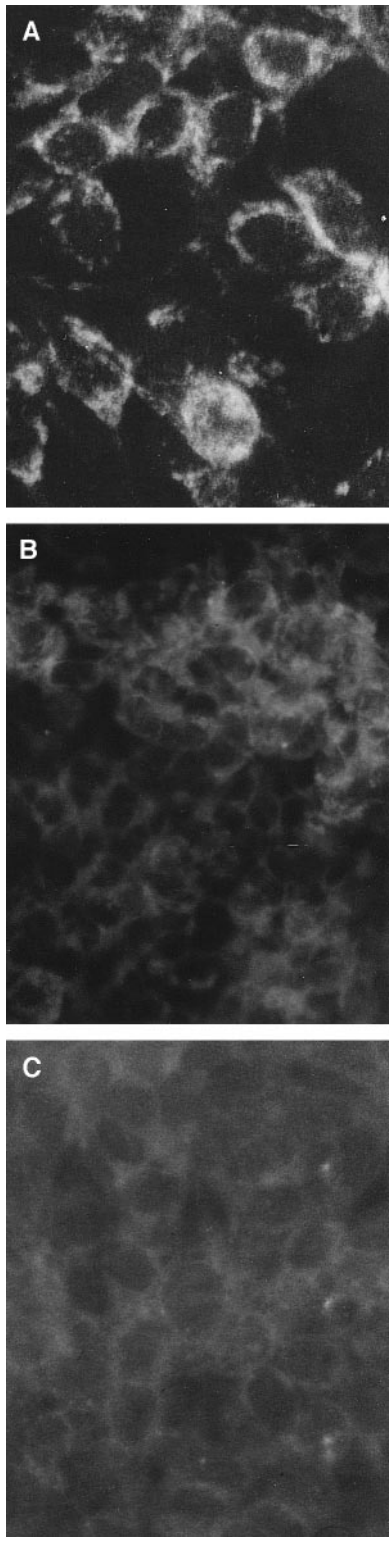

FIG. 1. Immunofluorescence localization of MuV HN or F using specific anti-HN or anti-F MAbs (kindly provided by Prof. J. Wolinsky) on Vero cells transfected with GC9/virosomes (A), GC23/virosomes (B), or pcDNA3 (C). Magnification ×740.

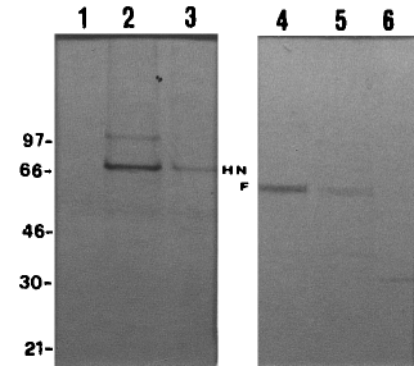

FIG. 2. Immunoprecipitation and SDS–PAGE analysis of the MuV HN and F antigens. Lanes 1 and 6 show mock-transfected cell extracts; lanes 2 and 4, cellular extracts from Vero cells infected with GC9/virosomes and GC23/virosomes; lanes 3 and 5, cellular extracts from cells transfected with naked GC9 and naked GC23, respectively. Samples were tested with anti-MuV HN MAbs (1, 2, and 3) or with anti-MuV F MAbs (4, 5, and 6). The molecular weight markers are shown on left (Amersham Life Sciences).

Influenza "priming" effect

Some groups of mice received the influenza priming because we wanted to mimic the epidemiological situation existing among humans. As shown in Table 1, the "priming" of mice with influenza vaccine (Swiss Serum and Vaccine Institute, Berne, Switzerland) and Escheriagen before immunization increased the humoral response of either IgG and s-IgA in the nasal cavity, increasing the antigen uptake and/or antigen presentation (De Haan *et al.,* 1996b; Douce *et al.,* 1995; Verweij *et al.,* 1998; Walker and Clements, 1993) (Table 1). Escheriagen is known to be a very potent mucosal immunogen and has also proved to be a strong adjuvant in humans (Glück *et al.,* 1999). In the present study this priming, administered intranasally, significantly stimulated IgG responses and induced a local s-IgA response in mice. It is

TABLE 1

Immunological Response of Mice Immunized by Intranasal Administration of MuV Vaccines at One Month after the Last Immunization

Mice	Serum IgG	BAL IgA	NW IgA
A (GC9)	NEG	NEG	NEG
B (GC9)[a]	268 ± 164	NEG	NEG
C (GC9/virosome)	35 ± 13	NEG	NEG
D (GC9/virosome)[a]	1132 ± 600	NEG	7 ± 3
E (GC23)	NEG	NEG	NEG
F (GC23)[a]	919 ± 437	NEG	NEG
G (GC23/virosome)	100 ± 43	NEG	NEG
H (GC23/virosome)[a]	1393 ± 358	NEG	NEG
I (virosome alone)	NEG	NEG	NEG
L (pcDNA)	NEG	NEG	NEG
M (live MuV)	2270 ± 1100	NEG	8 ± 2

Note. Animals were administered with 5 μg of DNA/virosome or with 5 μg of naked DNA ± Escheriagen. NEG, negative. The concentration of the specific IgA (μg/mg of total IgA) in each NW sample was normalized to the total IgA concentration, and reported as means ± SD. The IgG values are given as antibody GMT ± SD tested by ELISA.

[a] Group of mice that had received the influenza "priming."

interesting to note that, when priming was given with Escheriagen alone or with influenza virosomes alone, the adjuvant activity was suppressed. Moreover, Escheriagen did not show any adjuvant activity when administered with naked DNA (data not shown). Influenza HA and Escheriagen appear to be effective in our system only if they are administered together. The influenza priming effect has also been observed using virosomes as an antigen carrier system (Zurbriggen and Glück, 1999). It is possible that during the priming, Escheriagen supports the action of HA on the virosomes that bind macrophages and other immunocompetent cells through sialic acid-containing receptors. The mechanism of the influenza priming is not yet known. It is possible that the synergic effect of the two factors promotes the production of cytokines, which during the booster may have a positive influence on the DNA immunization. Consequently, the stimulated cells determine an enhanced immune response whenever virosomes associated with DNA plasmid are administered.

Humoral response to MuV antigens elicited by DNA immunization

The efficacy of intranasal (i.n.) immunization with plasmid DNA encoding the HN was evaluated at the mucosal site, with respect to the protocol of vaccination. Table 1 shows that i.n. immunization with DNA-virosomes after priming resulted in a significant stimulation of the serum IgG response with respect to the response induced by i.n. immunization with naked DNA ($P = 0.03$). It is generally assumed that the immunostimulating effect of liposomes primarily involves the uptake of liposome-associated antigen by cells of the mononuclear phagocytic system (De Haan *et al.,* 1996a; Lasic, 1998; McCluskie *et al.,* 1998; Wheeler *et al.,* 1996). In our study, the influenza HA on the virosomes facilitates the targeting of plasmid DNA to antigen-processing and -presenting cells. The increased uptake of DNA would result in an improved expression of the vaccine antigen (Templeton *et al.,* 1997). Virosomes may also improve transfection efficiency by increasing the retention time and reducing the rate of DNA degradation by extracellular nucleases (Dzau *et al.,* 1996; Meyer *et al.,* 1995). The MuV HN and F antigens are both capable of inducing specific anti-MuV antibodies. No cross-reaction was revealed between anti-MuV and anti-influenza antibodies as demonstrated by immunoenzymatic assays. The influenza priming had a positive effect on the humoral immune response of mice immunized with naked DNA (groups B and F), particularly with DNA-virosomes (groups D and H). These groups developed a response similar to that of mice immunized with the live MuV (Urabe Am9 strain) ($P = 0.09$).

This vaccine formulation also induced a neutralizing activity. The sera from mice which had received priming and were immunized with GC9 (MuV HN) developed a neutralizing response (GMT 8.3 $\pm$ 2.1) against the mumps virus, as did mice immunized with the live MuV (GMT 16). We did not find neutralizing antibodies in mice immunized with GC23. This result confirms other previously published data (Kövamees *et al.,* 1990; Örvell, 1984; Örvell *et al.,* 1997), which assess the presence of neutralizing epitopes in the mumps virus HN (Cusi *et al.,* submitted). The lack of an animal model suitable to show the MuV pathogenesis has slackened the molecular study of this virus. However, since the HN protein represents the major target to induce a protective immune response to mumps virus (Houard *et al.,* 1995), these results could be a step toward a genetically engineered vaccine against mumps virus infections.

Mucosal antibody response

By analyzing the mucosal immunity we found that, among the primed mice, only those immunized with GC9-virosomes developed an amount of IgA in NW samples comparable to the IgA level obtained in mice immunized with a high dose of live MuV ($P > 0.05$). Moreover, when mice were immunized i.n. with a lower dose of live MuV (1000 $TCID_{50}$/mouse), no IgA were detected in NW samples. However, this response was short-lived. IgA antibodies were also detected in feces drawn 7–10 days after the last immunization, indicating that i.n. administration of DNA also induces a mucosal immunity at the enteric site (data not shown) (Asakura *et al.,* 1997; Okada *et al.,* 1997). The presence of antibodies to the HN of mumps virus in the mucosa is very important, since they represent the barrier immunity and can prevent the binding of the virus to the specific receptor. It is unclear why mice immunized with naked GC23 or GC23-virosomes did not develop an IgA response; likely, the processing of the two MuV antigens, HN and F, inside the cells is different; in fact, they induce a different Th response in immunized animals, which could be responsible for promotion of IgA synthesis.

It is worth noting that IgA was revealed in the BALs only when mice had been immunized with GC9-virosomes and when the samples were drawn 2 weeks after the last immunization (Table 2). It is possible that a shorter-interval period between immunizations is necessary for inducing IgA in lungs, since the turnover is less than 1 week for bronchial epithelial cells; moreover, it has been suggested that alveolar macrophages suppress immune responses in lung by inhibition of T-cell proliferation and down-regulation of antigen-presenting functions of pulmonary dendritic cells (De Haan *et al.,* 1996a). The results demonstrate that virosomes administered with DNA have the capacity not only to induce IgG response but also to stimulate a significant local s-IgA response in the secretory tract.

TABLE 2

Immunological Response of Mice Immunized by Intranasal Administration of MuV Vaccines at 12 Days after the Last Immunization

Mice	Serum IgG	BAL IgA	NW IgA
A (GC9)	NEG	NEG	NEG
B (GC9)[a]	25 ± 7	NEG	NEG
C (GC9/virosome)	30 ± 5	8 ± 7	10 ± 4
D (GC9/virosome)[a]	356 ± 115	11 ± 6	10 ± 6
E (GC23)	NEG	NEG	NEG
F (GC23)[a]	22 ± 18	NEG	NEG
G (GC23/virosome)	28 ± 9	NEG	NEG
H (GC23/virosome)[a]	160 ± 110	NEG	NEG
I (virosome alone)	NEG	NEG	NEG
L (pcDNA)	NEG	NEG	NEG
M (live MuV)	1585 ± 566	20 ± 10	NEG

[a] Group of mice that had received the influenza "priming."

TABLE 3

Antigen-Specific Cytokine Production from Splenic Cells of Responding Groups of Mice following Influenza Priming and DNA Immunization

Group	IL-2	IFN-γ	IL-10
GC9	0	0	200
GC9-IRIV	0	0	50
GC23	500	0	0
GC23-IRIV	250	0	0
IRIV	0	0	0
pcDNA	0	0	0
live MuV	150	0	900

Note. Values are given in pg/ml. Cells were stimulated with 10 μg/ml of live MuV.

Cell-mediated immune response in immunized mice

Th play an important role in eliciting both humoral and cellular immune response (McDonnell and Askari, 1996; Shearer and Clerici, 1997; Tighe *et al.,* 1998); thus, T-cell proliferation was examined. Four weeks after the last immunization, splenocytes from mice were examined for proliferation in response to the specific antigen. Splenic lymphocytes responded when 10 μg/ml of purified MuV was used as antigen. As shown in Fig. 3, naked GC9 or GC23 plasmids induced a Th proliferation response higher than that of the negative control ($P = 0.02$). However, when GC9 or GC23 plasmids were associated with virosomes, Th cell proliferation levels were further enhanced. This can be explained by the fact that influenza virosomes have been shown to be powerful stimulators of T-cell proliferation (Saurwein-Teissl *et al.,* 1998). Similar results were also obtained regardless of whether mice had been primed before vaccination. In general, proliferation assays predominantly measure $CD4^+$ T-cell responses, a result supported by analyzing the pattern of cytokines that the subsets of Th cells produced (Parronchi *et al.,* 1991; Romagnani, 1991; Sher and Coffman, 1992). Antigen-specific cytokine production was not revealed as well as the anti-MuV IgG isotyping because of the low level of the specific IgG in mice of groups A, C, E, and G. Table 3 summarizes representative cytokine measurements of the responding animal groups (B, D, F, H, and M) obtained from two separate experiments.

Mumps virus-stimulated cells from mice inoculated with GC9-virosomes after the influenza priming induced the production of IL-10, whereas the production of IL-2 was induced in cells taken from mice immunized with naked GC23 and GC23-virosomes after priming. Cells from mice immunized with live MuV induced mainly IL-10 (800 ng/ml) and, to a lesser extent, IL-2 (150 pg/ml). IFN-γ was not revealed in any sample, although this result could be explained by the fact that the interferon system could be suppressed in mumps virus-infected cells (Yokosawa *et al.,* 1998; Hariya *et al.,* 1999). Table 4 shows the amount of specific IgG1/IgG2a immunoglobulins in the sera of mice drawn after i.n. administration of MuV vaccine or DNA-virosomes. The amount of IgG1 isotype was predominant in mice immunized with GC9-virosomes, whereas the amount of IgG2a isotype was predominant in mice immunized with GC23-virosomes. This result could be due to the fact that antibody isotype is determined not only by the method of immunization but also by the nature of both the antigen and the coimmunized antigen (Cardoso *et al.,* 1998). Intranasal immunization with live MuV induced IgG2a antibodies. Our results show that mucosal immunization with GC9-virosomes stimulates a Th2 response, since a higher production of IgG1 and a significant level of IL-10 were observed in immunized mice in addition to the presence of IgA Abs, which are typical indicators of this response (Wheeler *et al.,* 1996), while the IgG isotyping (increase of IgG2a level) and IL-2 production in mice immunized with GC23-virosomes characterize a Th1 response. Many factors in vaccine design can influence dominant cellular versus humoral responses, such as the antigen dose, the route of immunization, and the type of antigen. The presence of virosomes as a new delivery system associated with different antigens could contribute to directing a response toward dominant cellular or humoral

TABLE 4

Specific Anti-MuV IgG Isotyping

Group	IgG	IgG 1	IgG 2a
GC9	212	85.5	14
GC9-IRIV	323	105	9.5
GC23	163	8	84
GC23-IRIV	267	24.5	73.5
IRIV	0	0	0
pcDNA3	0	0	0
Live MuV	1862.5	235	1563

Note. Values are given in ng/ml.

immunity. The induction of mucosal IgA and neutralizing antibodies by immunization with GC9-virosomes could be very important to block the entry of MuV into the host. However, a combination of the two vaccines containing either the MuV HN or the MuV F proteins could represent a good candidate to obtain both humoral and cellular immune response.

A balanced Th1/Th2 cytokine profile, characterized by a significantly higher IL-10 response and the predominance of IgG2a Abs, was observed in mice immunized i.n. with the live MuV. In our study, HN and F proteins appear to stimulate a different Th reponse; thus, the presence of both of the antigens against the mumps virus could be necessary for a vaccine capable of inducing cellular and humoral immunity (Staats *et al.,* 1994).

Safety of the vaccine

Little is known about how long the antigen is produced after mucosal plasmid DNA administration. To address this issue, lung tissue and lymphomonocytes were examined by PCR to reveal the presence of the plasmid. Only 2% of the lymphocyte samples from the group of mice immunized with DNA-virosomes revealed the presence of plasmid DNA 1 month after the last immunization. No plasmid DNA was detected in lungs. It seems that lymphomonocytes could be the vehicle for DNA-virosomes. The persistence of the plasmid over a month after immunization could be useful for an adequate expression of the recombinant vaccine protein. It would be interesting to study the period of MuV genes expression after immunization. Studies have shown liposome-formulated DNA to be safe and nontoxic in animal models at doses producing immunological responses (Caplen *et al.,* 1995; Porteous *et al.,* 1997; Tsan *et al.,* 1997). Influenza virosomes were also shown to be nontoxic in mice; moreover, this system has the advantage of using as little as 5 μg of DNA to induce a good response and to provide an immune barrier at the mucosal sites, since it can be administered by the natural route of infection. It should also be important to transfer the technology from small animal models to nonhuman primates. The virosomes appear to be an effective tool for targeting and gene delivery (Cusi and Glück, 2000), providing a novel promising approach for the development of an efficacious human vaccine.

MATERIALS AND METHODS

Preparation of DNA plasmid:virosome complexes

The hemagglutinin (HN) and the fusion (F) genes of the Urabe Am9 strain of the mumps virus were amplified by RT-PCR (Cusi *et al.,* 1995, 1996), digested with *Bam*HI and *Bgl*II, respectively, and ligated into the pcDNA3 expression vector (InVitrogen, San Diego, CA) to obtain the recombinant plasmids GC9 (MuV-HN) and GC23 (MuV-F). Primers used for HN amplification were 5′-AACGGATCCAGATGGAGCCCTCGAAA-3′ and 5′-AGGGATCCTTATCAAGTGATAGTCAATCT-3′. Primers used for F amplification were 5′-ACAGATCTGATCAGTAATCATGAA-3′ and 5′-ACAGATCTTCAGGAGTTTACCTT-3′. The constructs were grown in DH5α cells and plasmid DNA was purified by Qiagen EndoFree plasmid Kit (Qiagen, Chatsworth, CA) as described by the manufacturer. Influenza virosomes were prepared as described elsewhere (Mengiardi *et al.,* 1995; Waelti and Glück, 1998). IRIVs are spherical, unilamellar vesicles which are prepared by a mixture of natural and synthetic phospholipids containing egg yolk phosphatidylcholine, phosphatidylethanolamine, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate (DOTAP), and 10% envelope phospholipids originating from influenza A/Singapore/6/86 and influenza surface glycoproteins. Briefly, 1 ml of DOTAP virosomes was added to 3.1 mg plasmid (1.3 μmol). Nonencapsulated plasmids were separated by gel filtration on a High Load Superdex 200 column (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was equilibrated with sterile PBS. The void volume fractions containing the virosomes with encapsulated plasmids were eluted with PBS and collected.

Transfection of Vero cells *in vitro*

About 10^5 Vero cells were grown on coverslips at 37°C and infected with 0.3 μg of DNA-virosomes or transfected with 1 μg of plasmid DNA using the Effectene Transfection reagent (Qiagen) as described by the manufacturer. After 2 days, mumps antigen expression was analyzed by immunofluorescence. The cells were washed twice with PBS, fixed with cold methanol/acetone, and treated with either anti-MuV HN or anti-MuV F MAbs (kindly provided by Prof. J. Wolinsky), followed by FICT-conjugated goat anti-mouse immunoglobulin G (1/100; Sigma, St. Louis, MO). The coverslips were mounted on slides and examined using a Diaplan microscope (Leitz, Wetzlar, Germany). Positive control (mumps virus-infected cells) and negative control (mock-infected cells) were included in each test.

Assessment of protein expression by RIPA

Vero cells (2×10^6) were plated onto a 35-mm tissue-culture dish, incubated for 1 h at 37°C, and transfected as described above. At 24 h after transfection, the cells were washed with PBS three times, incubated in methionine-free Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Life Technologies, MI, Italy) for 1 h at 37°C, and radiolabeled for 4 h at 37°C in methionine-free DMEM containing 100 μCi [^{35}S]methionine. The labeled cells were washed with PBS and lysed with the lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-P40 [NP-40]) at 4°C for 30 min. The cells debris was pelleted by centrifugation at 15,000 *g* for 5 min at 4°C. The lysate was mixed with 30 μl of anti-MuV HN or

anti-MuV F MAbs and 30 μl 50% protein A–Sepharose beads (Sigma) and incubated overnight at 4°C with rotation. The immune complexes were washed with SNNTE buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 0.5 M NaCl, 5% [w/v] sucrose, 1% [v/v] NP-40) and once with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton, 0.1% SDS, 1% deoxycholic acid). The precipitates were resuspended in sample buffer and boiled for 5 min. The same protein amount (≅10 μg) of each sample was then resolved by electrophoresis on an SDS–polyacrylamide gel. Following electrophoresis, the gel was fixed in isopropanol-acetic acid-dH_2O, fluorographed with Amplify reagent (Amersham Italia Srl, MI, Italy), dried, and exposed on Kodak X-Omat film at −70°C.

Immunization of mice

Four-week-old female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) were used. Each experiment ($n = 6$) was repeated three times to ensure the reproducibility of results. Mice were anesthetized with ketamine-xylazine and were immunized by i.n. instillation of 5 μg of DNA/mouse in a volume of 20 μl, resulting in deposition of the inoculum throughout the respiratory tract. Groups A–B and C–D were immunized with naked GC9 plasmid and GC9-virosomes complex, respectively; groups E–F and G–H were immunized with naked GC23 plasmid and GC23-virosomes complex, respectively. Booster immunizations were given 4 and 8 weeks after primary immunization. Groups B, D, F, and H received an intranasal priming with influenza virus vaccine (20 μl containing 0.6 μg of HA and 40 ng of Escheriagen) 10 days before the first immunization. Control groups consisted of mice administered virosomes alone (group I), or pcDNA3-virosomes (group L), or 5×10^5 $TCID_{50}$ of live mumps virus (Urabe Am9 strain) (group M). For collection of bronchoalveolar lavages (BAL) and nasal washes (NW), mice were terminated 2 weeks or 1 month after the last immunization by cervical dislocation under anesthetization. Collection of BALs and NWs from mice was performed as described elsewhere (Takao *et al.,* 1977).

ELISA

IgG and IgA antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Since the concentration of IgA in secretions is variable, the amount of MuV-specific IgA in NW samples was normalized to the total IgA concentration in each sample. Determination of total IgA was performed on flat-bottomed microtiter plates coated with 100 μl of goat anti-mouse IgA (1 μg/ml; Southern Biotechnology Associates, Birmingham, AL). Briefly, samples were diluted 1:50 and titrated in a series of threefold dilutions. After incubation at 4°C overnight, plates were washed three times and 100 μl of goat horseradish peroxidase-labeled anti-mouse IgA (1/6000; Southern Biotechnology Associates) was added. After incubation at 37°C for 3 h, 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) was added and allowed to react at room temperature for 30 min, and the reaction was stopped with 100 μl of 0.5 N H_2SO_4. Colorimetric conversion for the substrate was measured in a microplate spectrophotometer at 450 nm (Behring).

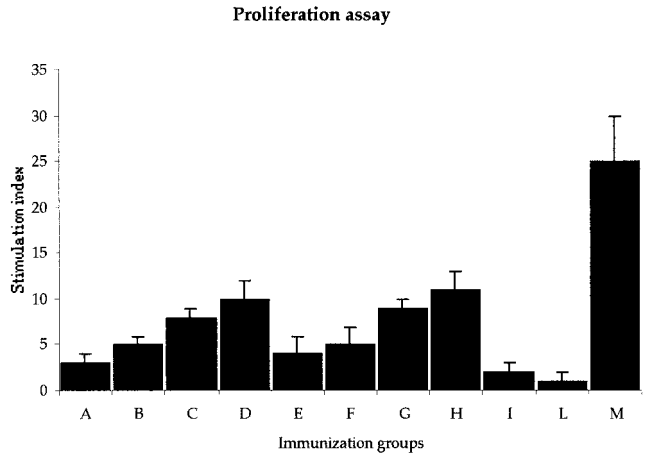

FIG. 3. T-cell proliferation responses following DNA immunization against MuV. Spleens were collected from mice immunized with GC9 (groups A–D), GC23 (groups E–H), or live MuV (Urabe Am9 strain) (group M), and their lymphocytes were isolated and tested for T-cell proliferation. Groups I and L represent the negative control of mice immunized with influenza virosomes and pcDNA3, respectively.

For the determination of mumps virus-specific IgG and IgA antibodies, purified virions of mumps virus were diluted in coating buffer (0.05 M $NaHCO_3/Na_2CO_3$, pH 9.6) to 1 μg of proteins per ml, and dispensed to a 96-well plate at 100 μl/well. After allowing them to absorb overnight at 4°C, the wells were washed with PBS–0.05% Brij 35 and blocked for preventing nonspecific binding by incubation with 5% heat-inactivated fetal calf serum (FCS) in PBS–Brij 35 for 2 h at room temperature. A 100-μl aliquot of samples was diluted twofold in the plate and allowed to react for 1 h at 37°C. The plate was then washed and 100 μl of goat horseradish peroxidase-labeled anti-mouse IGg (γ) antiserum (1/8000; Bio-Rad, Richmond, CA) for IgG ELISA or goat anti-mouse IgA (α) antiserum (1/6000; Southern Biotechnology Associates) for IgA ELISA was added and the plate was incubated for 1 h at 37°C. After washing, the substrate was added and allowed to react at room temperature for 30 min. Colorimetric conversion for the substrate was measured in a microplate spectrophotometer at 450 nm (Behring). IgG titers of samples were calculated from endpoint dilutions showing an optical density at least twice the value of the background represented by a pool of negative control sera. The concentration of total and MuV-specific IgA was calculated against a standard curve of mouse myeloma IgA (Cappel Laboratories, Cochranville, PA) determined on the same plate. Results were expressed as micrograms of MuV-specific IgA mg^{-1} of total IgA.

Neutralization assay

Virus neutralization was carried out on Vero cells in a 96-well microplate (Örvell *et al.,* 1997). Briefly, serial two-fold dilutions of immunized mice serum were added to an equal volume of mumps virus (wild type MuV strain, isolated and sequenced in the laboratory of the Microbiology Section, University of Siena) containing 100 $TCID_{50}$ in 50 μl and incubated for 90 min at 37°C. A 50-μl sample of cells (10^6/ml) was suspended in MEM with 5% FCS and added to each well. Five days after incubation at 37°C, the cultures were examined microscopically for the presence of the cytophatic effect.

Cytokine assay

Splenocytes were drawn from immunized mice and lymphocytes were collected by Ficoll–Hypaque (Pharmacia) gradient. About 100 μl of 2×10^6 unfractionated cells per ml in a complete RPMI 1640 plus 10% FCS were cultured in a total volume of 200 μl with 10 μg/ml of purified mumps virus or phytohemagglutinin (PHA, 5 μg/ml; Sigma) in a 96-well flat-bottomed plate. Control wells received cell suspension only. After 24 h in culture, cell-free supernatants were harvested for the presence of IL-2 and, after 48 h, for the presence of IFN-γ and IL-10. Samples were stored at −80°C. Briefly, microtiter plates were coated overnight at 4°C with 100 μl of anticytokine capture MAb (Pharmingen, San Diego, CA) at 1 μg/ml. The plates were washed twice with PBS–Tween and blocked with 100 μl of 10% FCS in PBS per well per 2 h at room temperature. The plates were then washed twice and incubated with duplicates of serially diluted samples and standards (Sigma) overnight at 4°C. Then 100 μl of the biotinylated anticytokines (Pharmingen) MAb at 1 μg/ml was added to each well and the mixture was incubated at room temperature for 1 h. The plates were then washed three times, 100 μl of streptavidin-peroxidase (1/1000; Sigma) was added, and the mixture was incubated at room temperature for 30 min. Following multiple final washings, the color was developed with TMB (Sigma) and stopped with 100 μl of 0.5 N H_2SO_4, and the absorbance at 405 nm was measured with an ELISA plate reader. The concentration of cytokines in samples was determined according to the standard curve.

Lymphocyte proliferation assay

To determine whether MuV lymphoproliferative responses were induced in immunized animals, their spleens were removed 4 weeks after the last immunization to make a single cell suspension. A 100-μl sample of splenocytes (2×10^6/ml) in complete RPMI 1640 was added to each well in 96-well flat-bottomed plates. Stimulated cells received purified MuV at a concentration of 10 μg/ml; transferrin served as a negative control (120 μg/ml; Sigma) and PHA (5 μg/ml; Sigma) as a positive control. Control wells received cells only. The cells in the well were cultured in 200 μl of medium. After 4 days in culture, the cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 18 h and harvested with FilterMate (Packard, Downers Grove, IL) and the incorporated radioactivity was determined by TopCount (Packard). The stimulation index was calculated as the mean cpm of the stimulated wells divided by the mean cpm of the control wells.

PCR

PCR was performed on DNA extracted from the lymphocytes and the lung tissue (2 mg) of immunized mice by using the QIAamp tissue Kit (Qiagen), as described by the manufacturer. Each sample was subjected to 40 cycles at 94°C for 1 min, 56°C for 40 s, and 72°C for 90 s. The primers used to detect GC9 were the sense 5′-TCCAGATGGAGCCCTCGAAA-3′ and the antisense 5′-TTATCAAGTGATAGTCAATCT-3′. The primers used to detect GC23 were the sense 5′-CCGCGATCAGTAATCATGAA-3′ and the antisense 5′-GCCGCTCAGGAGTTTACCTT-3′.

Statistical analysis

Antibody titers are presented as geometric means ± SD. The Mann–Whitney rank sum test was used to analyze changes in the level of total and specific IgG and IgA. $P < 0.05$ was considered significant.

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